Reprinted from THE JOURNAL OF PATHOLOGY AND BACTERIOLOGY, Vol. XXVII., 1924

The Fermentative Reactions of the Diphtheria Bacillus

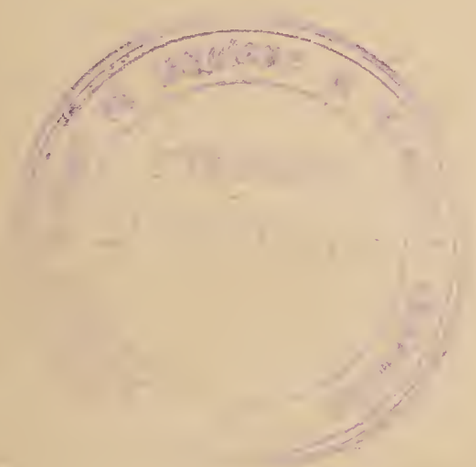
BY

C. C. OKELL, M.C., M.B. (Camb.), M.R.C.P. (Lond.)

AND

E. M. BAXTER, M.Sc. (Sheffield)

The Wellcome Physiological Research Laboratories



Reprinted from THE JOURNAL OF PATHOLOGY AND BACTERIOLOGY, Vol. XXVII., 1924

The Fermentative Reactions of the Diphtheria Bacillus

BY

C. C. OKELL, M.C., M.B. (Camb.), M.R.C.P. (Lond.)

AND

E. M. BAXTER, M.Sc. (Sheffield)

The Wellcome Physiological Research Laboratories

6582

THE FERMENTATIVE REACTIONS OF THE DIPHTHERIA BACILLUS.

C. C. Okell, M.C., M.B. (Camb.), M.R.C.P. (Lond.), and
E. M. Baxter, M.Sc. (Sheffield).

The Wellcome Physiological Research Laboratories.

In spite of the numerous papers that have been written from time to time on the fermentative reactions of *Coryne-bacterium diphtheriæ*, the subject can hardly be said to have yet reached finality and even in recent years there have been discrepancies in the results obtained by different authors. The excellent summary given in the recently published memoir on diphtheria of the Medical Research Council makes it unnecessary to discuss the literature in detail: table I. gives a summary.

Table I.

Author.	Date.	Glucose.	Sucrose.	Lactose.	Maltose.	Dextrin.	Glycerol.
Theobald Smith .	1896	...	O	O	...	...	...
L. Martin. . .	1898	A	A	O	O	...	A
Knapp . . .	1904	A	O	...	A	A	...
Zinsser . . .	1907	A	O	O	A	A	...
Rothe . . .	1907	A	O	O	A or O	...	...
Arkwright . .	1908	A	O	O	A	...	...
Graham-Smith . .	1908	A	O or A	O	A or O	A	...
Morse . . .	1912	A	O	...	A	A or O	A
Neisser and Gins .	1913	A	O	O	...	...	...
Hine	1913	A	O	A or O	A	A	...
Cary	1917	A	O	...	1 exception A or O	A or O	A
Guthrie, Gelien and Moss	1920	A	3 strains A O	...	...	...	...
Durand . . .	1921	A	O or A	O	A or O	A or O	A or O
Jordan and others .	1922	A	O	A or O	A	A, occasionally O	...
Fitzgerald and Doyle	1923	A or O	O or A	...	...	A or O	...
Barratt . . .	1923	A	O	O	A	A	...
Engering . . .	1923	A	O	A or O	A	...	...
Christiansen . .	1923	A	O	O	A	...	A
Present Series . .	1924	A	O	O	A	A	A

A = Acid production. O = No change.

Our excuse for the present contribution is that since our last note on this subject (Okell & Baxter, 1923) we have had occasion to examine the biochemical activities of a relatively large number of strains of the diphtheria bacillus which were checked throughout by virulence test in guinea-pigs. In carrying out this work we have kept the following special purposes in view: (*a*) to attempt to explain the ever-recurring discrepancies in the literature of the subject, (*b*) to attempt to separate virulent from avirulent strains by biochemical reactions, (*c*) to check the uniformity of the biochemical reactions of various serological groups of virulent *C. diphtheriæ*.

Source of cultures.

The cultures examined were from the throat, nose or ear of cases of diphtheria or of carriers.

Purification of cultures.

The cultures were provisionally diagnosed by a modification of Neisser's method and only those classified as "typical," "probably" or "possibly" *C. diphtheriæ* were submitted to further examination. The cultures were usually plated on Loeffler's medium, or in some cases when this medium was liquefied by the contaminating organisms present, on agar or Douglas's tellurite medium. The importance of careful isolation and purification cannot, we think, be too greatly emphasised. There seems to be little doubt that insufficient care in this respect has been responsible for some of the disagreements in the recorded results. We have found in confirmation of the observations of Zinsser (1907), Eagleton and Baxter (1922) and others that the purification of cultures of *C. diphtheriæ* may at times be no easy matter. Contamination may be so slight that one may fail to detect it in stained preparations, and yet so intimate that one can only attain purity by repeated platings. Staphylococci, streptococci and organisms of the *xerosis* group have proved the most troublesome contaminations, and frequently what appeared to be a quite discreet and typical colony of *C. diphtheriæ* contained a varying proportion of these contaminating organisms. In particular those cultures which ferment sucrose demand most careful examination before their purity can be vouched for. It is obvious that every type of diphtheria-like bacillus finally isolated from such a culture should be retested for virulence. The practical way of achieving this is by retesting several colonies from the plate.

Testing for virulence.

In the earlier work considerable confusion appears to have resulted from inadequate testing for virulence and even in some more recent papers it seems to have been taken for granted that all organisms resembling *C. diphtheriæ* isolated from cases of diphtheria should be taken as of ætiological significance. Avirulent organisms morphologically resembling *C. diphtheriæ* are, however, from time to time isolated from cases of diphtheria, though usually true virulent *C. diphtheriæ* can be isolated from the same culture. Very occasionally indeed it may happen that one isolates only such avirulent organisms from a case of diphtheria. Where two types of organisms resembling each other closely in morphology occur together, it is to be expected that at times one kind of colony only, and that perhaps the avirulent, should be selected from the plate. This has happened but rarely in our experience (twice in a series of 1891 cultures tested for virulence) and we have usually been able by replating from the original culture

or obtaining a new culture from the same patient, to isolate virulent *C. diphtheriæ* from cases of diphtheria. It should be mentioned that in four cases out of the total of 1891 strains tested by us for virulence we have isolated both a virulent and an avirulent *C. diphtheriæ* from the same throat or nose swab. This association is indeed so rare that we have not, as yet, been able to satisfy ourselves that its occurrence has been more frequent than could be explained by the laws of chance. These points are, we think, of importance since there is a distinct danger in ascribing ætiological significance to every organism morphologically resembling *C. diphtheriæ* that may be isolated from a case of diphtheria. We believe, however, that virulent *C. diphtheriæ* could be isolated in every case of true diphtheria had the bacteriologist reasonably free access to the patient.

All the cultures examined in the series reported here were tested for virulence in guinea-pigs. The method of virulence testing used was the intradermic method introduced by Römer (1909) and modified successively by Zingher and Soletsky (1915) and Eagleton and Baxter (1921). In the great majority of cases at least two colonies were tested for virulence. All reactions which gave rise to the least doubt in our minds were repeated, often several times. Many cultures were also tested by the subcutaneous method—the emulsifiable material of one Loeffler slope being injected into a normal and into a protected guinea-pig. Many avirulent strains were tested intradermically in tenfold the strength and without the "following dose" of antitoxin recommended by Eagleton and Baxter. No case of discrepancy was met with among the final results of these modifications of the virulence test.

Technique of the fermentation tests.

We found in common with several previous authors that certain of the organic materials used in the tests were damaged by such operations as autoclaving or steaming. We agree with Barratt (*Diphtheria*: Medical Research Council, p. 404) that unheated lactose is not fermented by *C. diphtheriæ* and that heated lactose may be fermented. In this investigation we have used reagents prepared in the following way:—

Double strength litmus broth is made according to the formula:—

100 c.c. nutrient broth	3 grms. Witte peptone
100 c.c. distilled water	2·1 grms. sodium chloride

and a sufficient quantity of litmus solution is then added to allow for the subsequent dilution with the sugar solutions. The P_H is then adjusted to 7·6 and test-tubes are filled out with about 2 c.c. quantities, plugged and autoclaved at 15 lbs. to the square inch for twenty minutes. Each tube filled out in this way is marked with a glass and china pencil so as to allow of the addition of an approximately equal volume of the sugar solution. The troublesome use of graduated pipettes is thus avoided. Solutions consisting of 0·5 per cent. to 1·0 per cent. of the sugar (according to the reagent) in distilled water are

filtered through a sterile Pasteur-Chamberland filter which has been previously tested. Each of the litmus broth tubes is then filled to the pencil mark with the sugar solution added with sterility precautions by means of a bulb pipette. The tubes of media are then incubated for forty-eight to seventy-two hours to ensure sterility and any tubes showing contamination are rejected. Such filtered reagents are quite easy to prepare and have given entirely consistent results in our hands.

Hiss's serum water was tried as a substitute for the broth, but on the whole we found that the broth basis gave a more satisfactory growth. It is of course important that the strains grow well on the media used for the tests, and on more than one occasion we found that the apparent inability of *C. diphtheriæ* to ferment glycerol, dextrin and milk was due to the difficulty of obtaining good growth on these reagents. The strains were always tested after plating, often after repeated platings. The growth from at least two colonies was always tested. Readings were made at the end of the first, fifth and tenth day of growth at 37° C. and sometimes also after fourteen and twenty-one days. When any inconsistency between two colonies from the same strain or any unexpected property in any strain appeared, the culture was replated and several colonies picked off and retested for virulence and fermentative properties. No inconsistency of any importance survived this process.

Results of the fermentation tests.

1. *Glucose and sucrose.*—By general consent the two most important sugars used in the identification of *C. diphtheriæ* are glucose and sucrose. Most authors are in agreement that it ferments the former and fails to ferment the latter. L. Martin (1898), Graham Smith (1908), Moshage and Kolmer (1916), Cary (1917), Durand (1921), Fitzgerald and Doyle (1923), however, claim to have found strains capable of fermenting sucrose. 430 cultures from our series singled out on morphology as "typical," "probably," or "possibly *C. diphtheriæ*" were examined for virulence. 200 of these were virulent to guinea-pigs and 230 avirulent. The 200 virulent strains, without exception, fermented glucose and failed to ferment sucrose. It should be pointed out that the figures given here have no bearing on the relative frequency of virulent and avirulent cultures since the cultures for biochemical investigation were selected arbitrarily from a much larger series of cultures, the virulence of which had been determined. When it was thought that a sufficient number of virulent strains had been examined, special attention was given to avirulent strains and an approximately equal number of these were investigated. Of the 230 avirulent strains, 200 fermented glucose and failed to ferment sucrose; 4 belonged to the *Hofmann* group, fermenting neither sugar, and 26 belonged to the *xerosis* group, fermenting both sugars. Thus, out of 230 arbitrarily chosen avirulent strains of diphtheria-like organisms, all but 30 showed the typical reactions of virulent *C. diphtheriæ* in

glucose and sucrose. We think that from the practical point of view it is useful to define the avirulent *C. diphtheriæ* as an organism morphologically indistinguishable from virulent *C. diphtheriæ* which ferments glucose, fails to ferment sucrose and which produces no specific (diphtheria) toxin either *in vivo* or *in vitro*.

2. *Other "sugars."*—Among the 430 strains examined, 104 virulent strains and 100 avirulent strains were tested on a more extended range of reagents. The sources of the 104 virulent strains were as follows: 49 from cases of diphtheria, 38 from contacts and "carriers"; the remaining 17 were laboratory strains of uncertain origin. 76 were from the throat, 10 from the nose, and 1 from the ear of a case of diphtheria, complicated by otitis media. Of the 100 avirulent strains, 1 came from a case of diphtheria, 98 from contacts or "carriers" and 1 from a case of otitis media. 65 were from the throat, 34 from the nose, 1 from the ear. The results of these tests are shown in table II. Owing to the fact that from time to time we gave special attention to certain reactions, all the strains were not submitted to every reagent in the series. Where a blank occurs in the table the strains were not tested against the reagent indicated.

Table II.

	Glucose.	Sucrose.	Lactose.	Glycerol.	Maltose.	Galactose.	Dextrin.	Litmus milk or whey.
				104 *virulent strains.*				
21	A	O	O	A	...	...	A	A or O
35	A	O	O	A	A	A	A	,,
28	A	O	...	...	A	A	A	,,
20	A	O	O	A	...	...	...	,,
104								
				100 *avirulent strains.*				
20	A	O	O	A	A	A	A	A or O
42	A	O	...	...	A	A	A	,,
32	A	O	O	...	A	A	A	,,
6	A	O	...	...	A	A	O	,,
100								

A = acid production. O = no change.

Included in the 35 virulent cultures which were tested with all the reagents were examples of the 10 serological types of virulent *C. diphtheriæ* described by Eagleton and Baxter as well as 16 of their unclassified cultures. All these gave exactly the same reactions.

By the courtesy of Dr W. H. Park of New York, we were able to obtain the type cultures stated to have been used by Durand in his

serological work on *C. diphtheriæ*. Durand (1921) states that one of his type organisms was a sucrose fermenter. All these cultures gave exactly the same reactions on the range of reagents indicated in table II. with the exception of one which fermented both glucose and sucrose. This was the only one of the cultures which proved avirulent to the guinea-pig. It should therefore, we think, be considered as belonging to the *xerosis* group rather than to the diphtheria group.

3. *Constancy of fermentation reactions.*—Examples of the seven most important serological groups of *C. diphtheriæ* described by Eagleton and Baxter (1923) were submitted to a further range of reagents at intervals during two years. The strain "Park Williams 8" (type I—Eagleton and Baxter) was included in this representative selection. The results of these tests are shown in table III. Two avirulent organisms were also included. In every case the fermentation reactions were found to be entirely regular and consistent. They were re-examined on the sugars indicated in table II. after intervals of subculture of eighteen, twenty-three and thirty-three months and showed no variation in their reactions during those periods.

TABLE III.

Glucose	A	Mannite	O
Sucrose	O	Adonite	O
Lactose	O	Raffinose	O
Dextrin	A sometimes slight.	Arabinose	O
Glycerol	A sometimes slight.	Inulin	O
Fructose	A	Salicin	O
Galactose	A	Amygdalin	O
Maltose	A sometimes slight.	Arbutin	O
Dulcite	O	Erythrol	O

Litmus milk	slight A or O, no coagulation.
Hiss's serum water	no coagulation.
Neutral red broth	no fluorescence.
Peptone water	no indol (by Ehrlich's reagent).

It will be seen that no strain of virulent or avirulent *C. diphtheriæ* fermented unheated lactose. Representatives of the 10 serological types and 8 avirulent strains were tested with five different commercial preparations of lactose including a pre-war preparation of Kahlbaum's and all the strains proved negative to lactose with all the samples. Dextrin was fermented by all the virulent strains tested, though occasionally only slightly. Only 6 out of 100 avirulent strains failed to ferment this reagent. We could find no evidence in favour of Hine's (1913) suggestion that dextrin fermentation is of value in the recognition of the true diphtheria bacillus. The difficulty of determining the exact chemical nature of "bacteriological" dextrin is notorious. Organisms of the 10 virulent serological types and 8 avirulent organisms, were tested with four reputable brands of dextrin including a pre-war preparation of Kahlbaum's. All samples were apparently free from

maltose. All the samples were fermented, some more markedly than others. Three different preparations of maltose and three of galactose gave consistent results with the same eighteen strains. Glycerol always became acid though sometimes only slightly so. Salicin was not fermented by any strain tested. Litmus milk proved an unsatisfactory reagent. The change produced by both virulent and avirulent strains was seldom marked and sometimes undetectable. There was, however, a general tendency to slight acid formation. Neutral red whey proved no more satisfactory. The hydrogen-ion concentration of whey was determined at intervals in the case of 10 virulent and 8 avirulent strains. The P_H fluctuated only within relatively narrow limits showing a slight acid change during the first few days, while at the end of two weeks a reverse change towards the alkaline side of the original reaction of the medium was manifested with the majority of strains.

Thus, with regard to the virulent strains, we find ourselves in entire agreement with Zinsser (1907) who found that "there were no exceptions and no irregularities" in the fermentation of the 42 strains he examined. In the case of the avirulent *C. diphtheriæ* (*i.e.*, organisms morphologically resembling virulent *C. diphtheriæ*, fermenters of glucose and non-fermenters of sucrose), we found a large number that were indistinguishable from virulent *C. diphtheriæ* except by virulence tests. There was therefore no evidence that the fermentation of the customary reagents was of any value in distinguishing virulent from avirulent strains.

It must be remembered that all our material came from the throat, nose or ear of human beings, and consisted solely of organisms morphologically indistinguishable (by Neisser's stain) from *C. diphtheriæ*. Barratt (1923) made a very extensive study of the diphtheroid group (which included diphtheroids from numerous sources many of which were excluded on morphological grounds from the group we examined). Her group of 17 strains of non-virulent *C. diphtheriæ* all gave reactions exactly similar to those we have found in the group we examined. The strains with which she worked were derived partly from ear, nose and throat, and partly from other sources, and it is pointed out that certain strains which might, on morphological grounds and failure to ferment sucrose, be placed in the group of non-virulent *C. diphtheriæ*, may be separated therefrom by failure to ferment galactose. These are relegated to groups III. and IV. (M.R.C. Memoir, p. 411). In a personal communication Dr Barratt informs us that about 16 per cent. of diphtheroids from ear, nose and throat (122 examined) fall into groups III. and IV. characterised by failure to ferment galactose. Maltose, she agrees, is of very minor value.

When dealing with strains from throat and nose we have been unable to find maltose and galactose of any value in the further classification of what we have previously defined as avirulent *C. diphtheriæ*. The difference of opinion as to the value of galactose between Dr Barratt and ourselves is, we think, only an apparent one.

Dr Barratt was intentionally examining a wider group than we, and her dividing line in determining which cultures on morphological criteria could be regarded as closely allied to the true *C. diphtheriæ* was fixed at a slightly different point from the one we adopted. In other words, the strains in her groups III. and IV., separated only by failure to ferment galactose, either did not occur in our series or, if they occurred, were regarded by us as morphologically not sufficiently near to the true *C. diphtheriæ* to justify, for the purpose of our research, their inclusion amongst what we were calling potential avirulent *C. diphtheriæ*.

Dextrin alone allowed of the separation of a small group of six which failed to ferment this reagent. Taking into consideration the uncertain chemical nature of "dextrin" and also the fact that the strains which failed to ferment it usually grew badly on the medium, we doubt whether this group were really non-fermenters. Indeed, with cultures from the sources indicated we have found that the only reagents of practical importance in differentiating the diphtheria from the diphtheroid groups are glucose and sucrose. Thus from the results of this work it would appear that the fermentative reactions of both virulent and avirulent *C. diphtheriæ* are of great constancy, and we can only explain the numerous disagreements in the recorded work on the subject as due to neglect of one or other of the precautions we have indicated as necessary to obtain consistent results.

In conclusion we wish to acknowledge our thanks to Dr Forbes and Dr Caiger who have provided us with many carefully described cultures and to Dr Eagleton who has given us much help.

Summary.

1. All diphtheria bacilli examined had consistent fermentation reactions. Many serological groups were included in the examination.

2. All virulent diphtheria bacilli tested (200 strains) fermented glucose and failed to ferment sucrose.

3. 200 out of 230 strains of avirulent bacilli picked out as indistinguishable morphologically from virulent diphtheria bacilli fermented glucose and failed to ferment sucrose.

4. The following explanations of the discrepancies occurring in the literature are suggested:—

(*a*) insufficient precautions as to the purity of the cultures;

(*b*) insufficient separation of the virulent from the avirulent strains;

(*c*) insufficient care in the preparation of the reagents, particularly of the di- and poly-saccharides which readily hydrolyse on heating;

(*d*) insufficient care to ascertain that free growth occurs in the media used.

5. Virulent could not be distinguished from avirulent diphtheria bacilli by means of any of the fermentation reactions tried.

REFERENCES.

ARKWRIGHT, J. A. . . . . *J. Hyg.*, 1908, viii. 48.

BARRATT, M. M. . . . . Diphtheria (Medical Research Council Memoir), *London*, 1923, p. 401.

CARY, W. E. . . . . *Journ. Infect. Dis.*, 1917, xx. 244.

CHRISTIANSEN, M. . . . . Le Bacille de la Diphtérie, 1923, *Paris*, p. 126.

DURAND, P. . . . . *Compt. rend. Soc. de Biol.*, 1921, lxxxiv. 982.

EAGLETON, A. J., AND BAXTER, E. M. *Brit. Med. Journ.*, 1921, i. 775.

EAGLETON, A. J., AND BAXTER, E. M. *Brit. Med. Journ.*, 1922, i. 139.

EAGLETON, A. J., AND BAXTER, E. M. *Journ. Hyg.*, 1923, xxii. 107.

ENGERING, P. . . . . *Centralbl. f. Bakteriol.*, 1922-23, 1 Abt., Orig. lxxxix. 120.

FITZGERALD, J. G., AND DOYLE, DOROTHY G. *Trans. of Royal Soc. of Canada*, 17, Third Series, 1923, p. 93; also *Journ. Amer. Med. Ass.*, 1923, lxxx. 1675.

GRAHAM SMITH, G. S. . . . The Bacteriology of Diphtheria. Nuttall and Graham Smith, 1908, *Cambridge*, 1913, p. 122.

GUTHRIE, C. G., GELIEN, J., AND MOSS, W. L. *Johns Hopkins Hosp. Bull.*, 1920, xxxi. 388.

HAVENS, L. C. . . . . *Journ. Infect. Dis.*, 1920, xxvi., 388.

HINE, T. G. M. . . . . This *Journal*, 1913-14, xviii. 75.

JORDAN, J. H., SMITH, FLORENCE, AND KINGSBURY, A. N. *Lancet*, 1922, ii. 1052.

KNAPP, A.. . . . . *Journ. Med. Research*, 1904, xii. 475.

MARTIN, L. . . . . *Ann. de l'Inst. Pasteur*, 1898, xii. 26.

MOSHAGE, EMILY L., AND KOLMER, J. A. *Journ. Infect. Dis.*, 1916, xix. 19.

MORSE, MARY . . . . . *Journ. Infect. Dis.*, 1912, xi. 253.

NEISSER, M., AND GINS, H. A. Ueber Diphtherie. Handb. der pathog. Mikroorganismen, Kolle-Wassermann, Jena 2te Aufl., 1913, v. 952.

OKELL, C. C., AND BAXTER, E. M. *Lancet*, 1923, i. 436.

RÖMER, P. H. . . . . *Ztschr. f. Immunitatsforsch.*, 1909, Orig. i. 175.

ROTHE . . . . . *Centralbl. f. Bakteriol.*, 1907, 1 Abt., Orig. xliv. 618.

SMITH, THEOBALD . . . . . *Tr. Ass. Amer. Physicians*, 1896, xi. 37.

ZINGHER, A., AND SOLETSKY, D. *Journ. Infect. Dis.*, 1915, xvii. 454.

ZINSSER, H. . . . . *Journ. Med. Research*, 1907-8, xvii. 277.

PRINTED BY OLIVER AND BOYD, EDINBURGH